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High-titer recombinant adeno-associated virus production utilizing a recombinant herpes simplex virus type I vector expressing AAV-2 Rep and Cap

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Recombinant adeno-associated virus type 2 (rAAV) vectors have recently been used to achieve long-term, high level transduction in vivo. Further development of rAAV vectors for clinical use requires significant technological improvements in large-scale vector production. In order to facilitate the production of rAAV vectors, a recombinant herpes simplex virus type I vector (rHSV-1) which does not produce ICP27, has been engineered to express the AAV-2 rep and cap genes. The optimal dose of this vector, d27.1-rc, for AAV production has been determined and results in a yield of 380 expression units (EU) of AAV-GFP produced from 293 cells following transfection with AAV-GFP plasmid DNA. In addition, d27.1-rc was also efficient at producing rAAV from cell lines that have an integrated AAV-GFP provirus. Up to 480 EU/cell of AAV-GFP could be produced from the cell line GFP-92, a proviral, 293 derived cell line. Effective amplification of rAAV vectors introduced into 293 cells by infection was also demonstrated. Passage of rAAV with d27.1-rc results in up to 200-fold amplification of AAV-GFP with each passage after coinfection of the vectors. Efficient, large-scale production (>10^9 cells) of AAV-GFP from a proviral cell line was also achieved and these stocks were free of replication-competent AAV. The described rHSV-1 vector provides a novel, simple and flexible way to introduce the AAV-2 rep and cap genes and helper virus functions required to produce high-titer rAAV preparations from any rAAV proviral construct. The efficiency and potential for scalable delivery of d27.1-rc to producer cell cultures should facilitate the production of sufficient quantities of rAAV vectors for clinical application.

Keywords: *adeno-associated virus; herpes simplex virus; rAAV; gene therapy; vector production*

Introduction

Recombinant adeno-associated virus type 2 vectors (rAAV) have been extremely successful vectors for *in vivo* gene transfer. These vectors have produced long-term, high-level gene expression of therapeutic proteins in immunocompetent animal models. For example, sustained production of erythropoietin from skeletal muscle after rAAV transduction has been achieved in mice.[1] Therapeutic levels of factor IX have been produced after rAAV gene transfer to the liver and skeletal muscle.[2–5] Levels of therapeutic protein production have reached up to 800 μg/ml in mice treated intramuscularly with AAV vectors expressing alpha-1 antitrypsin.[6] Recombinant AAV vectors have been used effectively in the central nervous system.[7–9] In addition, rAAV has been used in human clinical trials to transfer the CFTR gene.[10]

Production of sufficient quantities of high-titer rAAV needed for effectiveness *in vivo* has been difficult to achieve, however. The process requires the efficient cellular delivery of the proviral construct to be packaged as rAAV, the AAV-2 *rep* and *cap* genes, as well as specific helper virus functions.[11] The proviral construct to be packaged contains the cDNA expression cassette flanked by AAV-2 inverted terminal repeats (ITRs). The ITRs are the *cis* acting viral DNA sequences required to direct replication and packaging of the rAAV vector.[12,13] AAV-2 *rep* and *cap* genes encode the four Rep proteins (Rep 78, 68, 52 and 40) involved in viral DNA replication, resolution of replicative intermediates and generation of single-strand genomes and the three structural genes (VP1, VP2 and VP3) that make up the viral capsid.[14–16] Usually, the proviral rAAV and the *rep* and *cap* genes are introduced into cells by plasmid transfection. Replication and packaging of rAAV then occurs after expression of specific genes from a helper virus such as adenovirus (Ad).[14,17–20] Traditionally, Ad infection is used to provide helper virus functions.[11] In the case of Ad, the specific helper functions have been identified as the E1a, E1b, E2a, E4orf6 and Va RNA genes. These Ad genes encode proteins or RNA transcripts which are transcriptional regulators and are involved in DNA replication or modify the cellular environment in order to permit efficient viral production.[14,17–20]

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Recent improvements in rAAV packaging technology have made production of high-titer rAAV more feasible. One significant advancement has been the development of an Ad-free method for rAAV production.[20,21] This method is based on transfection of a plasmid encoding the Ad helper functions required for the production of rAAV. Other improvements have included the generation of *rep* inducible cell lines, translational control of Rep production and increasing Cap expression by driving *cap* transcription with a strong heterologous promoter.[22–24] These improved methods still possess limitations, however. The *rep* inducible cell lines do not produce rAAV more efficiently than traditional methods. Translational and transcriptional control of Rep and Cap production do not increase the efficiency of rAAV production more than 10-fold.[23,24] The Ad free method requires successful transfection on a large scale that is not easily achieved.

While Ad is an efficient helper virus for rAAV production, little consideration has been given to other helper viruses for AAV-2 replication and packaging. Herpes simplex virus type 1 (HSV-1) is also a fully competent helper virus of AAV-2.[25–28] The minimal set of HSV-1 genes required for AAV-2 replication and packaging has been identified as the early genes UL5, UL8, UL52 and UL29.[28] These genes encode components of the HSV-1 core replication machinery – the helicase, primase and primase accessory proteins and the single-stranded DNA binding protein (reviewed in Refs 29 and 30).

We have investigated the use of a recombinant HSV-1 (rHSV-1) vector to facilitate production of rAAV. A rHSV-1 has been engineered to express the AAV-2 *rep* and *cap* genes (*d27.1-rc*). This rHSV-1 vector, *d27.1-rc*, does not produce ICP27, a protein required for HSV-1 replication. Although *d27.1-rc* is replication defective, it does express the HSV-1 early genes required for rAAV replication and packaging.[28,31]

The vector *d27.1-rc* has been found to be as efficient at producing rAAV as Ad-free methods and obviates the need for large-scale transfection protocols. In addition, the rHSV-1 vector is 100 times more efficient at producing rAAV than previously described amplicon system based on the HSV-1 helper functions.[32] The rHSV vector, *d27.1-rc*, is a novel, flexible and simple way to introduce the AAV-2 *rep* and *cap* genes and helper virus functions required to produce high-titer rAAV preparations. The potential for scaleable rAAV growth using *d27.1-rc* should facilitate production of sufficient quantities of rAAV vectors required for clinical applications.

Results

Construction and characterization of d27.1-rc

The rHSV-1, *d27.1-rc* was constructed by homologous recombination of the AAV-2 *rep* and *cap* genes into the *tk* locus of the rHSV-1 virus *d27.1* (Figure 1). In this recombinant virus, the AAV-2 *rep* and *cap* genes are under control of their native promoters – the p5, p19 and p40 promoters. The p5, p19 and p40 promoters drive expression of the AAV-2 proteins Rep 78 and 68, Rep 52 and 40 and the capsid structural proteins VP1, VP2 and VP3, respectively.[33–37] Homologous recombination into the *tk* gene was confirmed by Southern blot analysis of restriction digests of *d27.1-rc* infected cell DNA (data not shown). In addition, *d27.1-rc* plaque formation on V27 cells, a complementing cell line, was not affected by 5-bromo-deoxycytidine. This indicates that the *tk* gene, appropriately, did not produce functional thymidine kinase (data not shown).

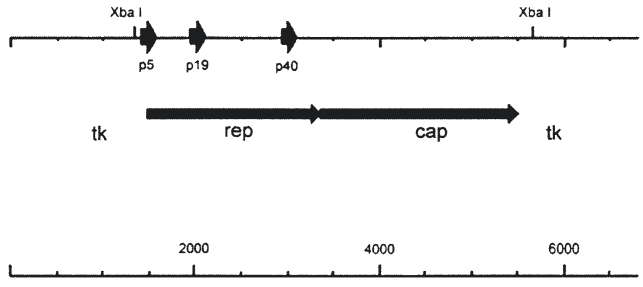

Figure 1 *Schematic of the integration vector pHSV-106-rc. The plasmid pHSV-106 contains the BamHI fragment encoding the tk gene of HSV-1. The AAV-2 rep and cap genes, under control of their native promoters, were cloned into the KpnI site of tk gene to generate pHSV-106-rc. Restriction digest of pHSV-106-rc with SphI was used to generate the linear fragment. This fragment was cotransfected with d27.1-lacZ infected cell DNA into V27 cells to generate d27.1-rc by homologous recombination.*

Production of AAV-2 Rep by d27.1-rc

In order for *d27.1-rc* to compliment rAAV replication, the AAV-2 Rep proteins must be efficiently expressed and localized to the nucleus of the cell after *d27.1-rc* infection. To determine the level of expression of the AAV-2 Rep proteins from *d27.1-rc*, Western analysis was utilized. The expression of the AAV-2 Rep proteins from *d27.1-rc* after infection of three different cell lines (293, Vero and V27 cells) at different multiplicities of infection (MOI) was analyzed (Figure 2).

The vector *d27.1-rc* expressed different levels of each of the AAV-2 Rep proteins in the different cell lines (Figure 2). In 293 cells, high level expression of all four Rep proteins occurred after infection with *d27.1-rc.* Expression of the Rep proteins was also observed in Vero cells after *d27.1-rc* infection. In contrast, only a small amount of Rep was produced in V27 cells after *d27.1-*

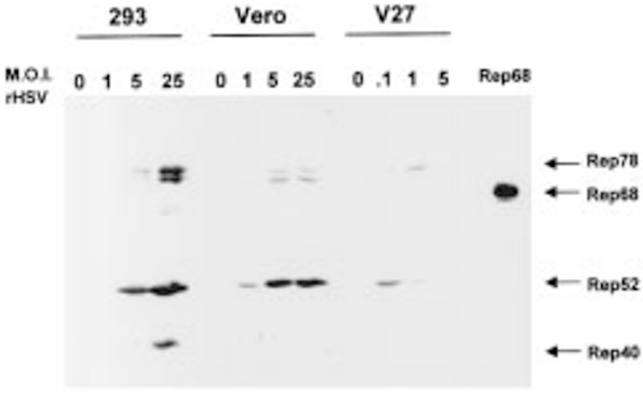

Figure 2 *Western analysis was performed to determine the expression of the AAV-2 Rep proteins from d27.1-rc. The expression of the AAV-2 Rep proteins from d27.1-rc after infection of three different cell types (293, Vero and V27 cell lines) at different MOIs was determined. The highest level of Rep expression was observed in 293 cells. Rep expression was also detected in Vero cells. Minimal Rep expression was observed in V27 cells, especially at higher MOIs.*

rc infection, especially at higher MOIs. The level of Rep expression after *d27.1-rc* infection of 293 and Vero cells was observed to be dependent on the MOI. The higher level expression of Rep in 293 cells after *d27.1-rc* infection may be due to up-regulation of the p5 promoter by Ad E1a present in 293 cells. The low level of Rep expressed in V27 cells after *d27.1-rc* infection in part results from lytic replication of *d27.1-rc* after infection of this cell line.

The Rep produced by d27.1-rc localizes to the nucleus

The cellular distribution of the AAV-2 Rep proteins was determined in an immunofluorescence assay (IFA) which utilized a monoclonal antibody that recognizes the four Rep proteins. The Rep proteins, expressed after infection of 293 cells by *d27.1-rc*, localized to discrete nuclear punctate bodies (Figure 3a). The distribution of Rep proteins to the nucleus of 293 cells infected with *d27.1-rc* is a prerequisite for rAAV replication.

Replication center formation by d27.1-rc

The observation has been made that the *rep* gene products are capable of inhibiting viral and cellular DNA replication.[38–40] In particular, *rep* gene products have been shown to be potent inhibitors of Ad DNA replication and prevent the maturation of Ad DNA replication centers.[41] This inhibitory effect of Rep proteins is presumably responsible for the inability to generate a recombinant Ad that expresses the AAV-2 *rep* gene. If *rep* gene products similarly inhibited HSV-1 viral DNA replication, the recombinant virus, *d27.1-rc*, would not be able to propagate. Replication of *d27.1-rc* was not affected by the presence of the *rep* gene, however. The kinetics of plaque formation on V27 cells, the complementing cell line, and the amount of virus produced per cell was identical to the parent virus, *d27.1* (data not shown).

In addition, the development of HSV-1 DNA replication centers after *d27.1-rc* infection of V27 cells was not affected by the presence of the *rep* gene. HSV-1 replication centers develop in the nuclei of infected cells in a time-dependent manner.[42] Viral and cellular proteins required for viral DNA replication (such as the HSV-1 core replication proteins which include ICP8, the single-stranded DNA binding protein) and replicating viral DNA localize to these centers.[42–45] Mature HSV-1 replication centers were observed in the nuclei of V27 cells 12 h after *d27.1-rc* infection, as indicated by the distribution of ICP8 (Figure 4a). This distribution of ICP8 is characteristic of fully developed HSV-1 replication centers[45] and did not differ from replication centers formed in V27 cells by the parent virus, *d27.1* (data not shown). In addition, minimal AAV-2 Rep expression was observed in V27 cells after *d27.1-rc* infection (Figure 4b).

The vector d27.1-rc is efficient at producing infectious rAAV from different rAAV proviral templates

To determine the flexibility and efficiency of rAAV production using *d27.1-rc*, we tested the production of rAAV from proviral plasmid transfected into cells, from a proviral cell line and by amplifying rAAV by coinfection. The vector *d27.1-rc* was observed to effectively rescue rAAV from pTR-UF5-transfected 293 cells. The plasmid pTR-UF5 contains a proviral rAAV genome that encodes the green fluorescent protein (GFP).[46] Transfection of 293 cells with pTR-UF5 followed by super-infection with *d27.1-rc* resulted in rescue of infectious AAV-GFP (Figure 5a and b). The amount of AAV-GFP produced was a function of the MOI of *d27.1-rc*. An increase in the yield of AAV-GFP was observed up to an MOI of 10. At this MOI, the yield of AAV-GFP was 381 expresion units (EU) per cell. This level of production compares favorably with recently developed rAAV production protocols based upon Ad-free transfection procedures.[20,21] Infection of pTR-UF5-transfected 293 cells with a control virus, *d27.1-lacz*, at an MOI of 10 did not produce AAV-GFP (data not shown).

The vector *d27.1-rc* was also capable of efficient AAV-GFP production from the cell line GFP-92 (Figure 6). In the cell line GFP-92, a proviral rAAV genome that encodes GFP is integrated into the chromosomal DNA. As in the transfection experiment, the amount of AAV-GFP produced was observed to be a function of the MOI of *d27.1-rc*. At the most efficient MOI for AAV-GFP replication and packaging, 480 EU/cell could be produced using the vector *d27.1-rc*. Infection of this cell line with the control virus *d27.1-lacz* at an MOI of 10 did not produce AAV-GFP (data not shown).

Amplification of rAAV via co-infection with rHSV

Interestingly, *d27.1-rc* can also be used to amplify rAAV genomes introduced into cells by infection of rAAV

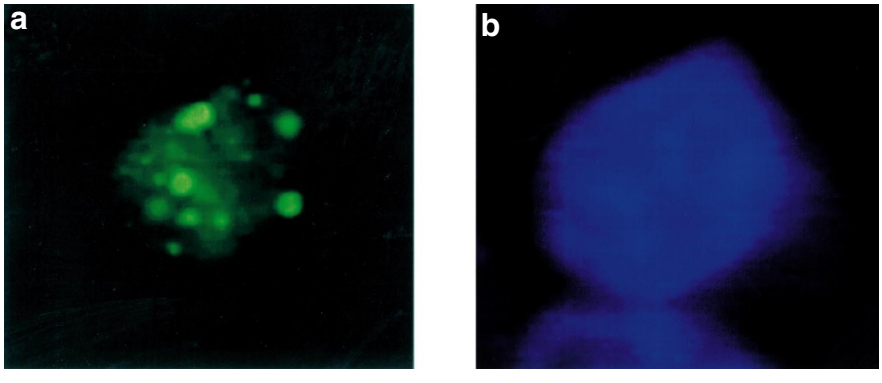

Figure 3 *(a) Immunofluorescence assay detecting the distribution of the AAV-2 Rep proteins, 10 h after infection of 293 cells with d27.1-rc. The 293 cells were processed for IFA and the cells were incubated with a monoclonal antibody that detects all four Rep proteins (78, 68, 52 and 40). The cells were then incubated with a FITC-conjugated, donkey anti-mouse secondary antibody. Magnification ×630. (b) DAPI counterstain of the nucleus in (a). Magnification ×630.*

Figure 4 *Immunofluorescence assay showing the development of mature HSV-1 viral DNA replication centers and minimal Rep expression in V27 cells after infection with d27.1-rc. Twelve hours after infection (MOI of 1), V27 cells were processed for IFA and incubated with a rabbit, anti-ICP8 antibody and a monoclonal, anti-Rep antibody. The cells were then incubated with a rhodamine-conjugated, donkey anti-rabbit secondary antibody and a FITC-conjugated, donkey anti-mouse secondary antibody. (a) Distribution of ICP8 in V27 cells infected with d27.1-rc. The observed nuclear distribution of ICP8 is characteristic of mature HSV-1 replication centers. Magnification ×630. (b) Rep expression is not detected in V27 cells infected with d27.1-rc. Magnification ×630. (c) DAPI counterstain of the nuclei in (a) and (b). Magnification ×630.*

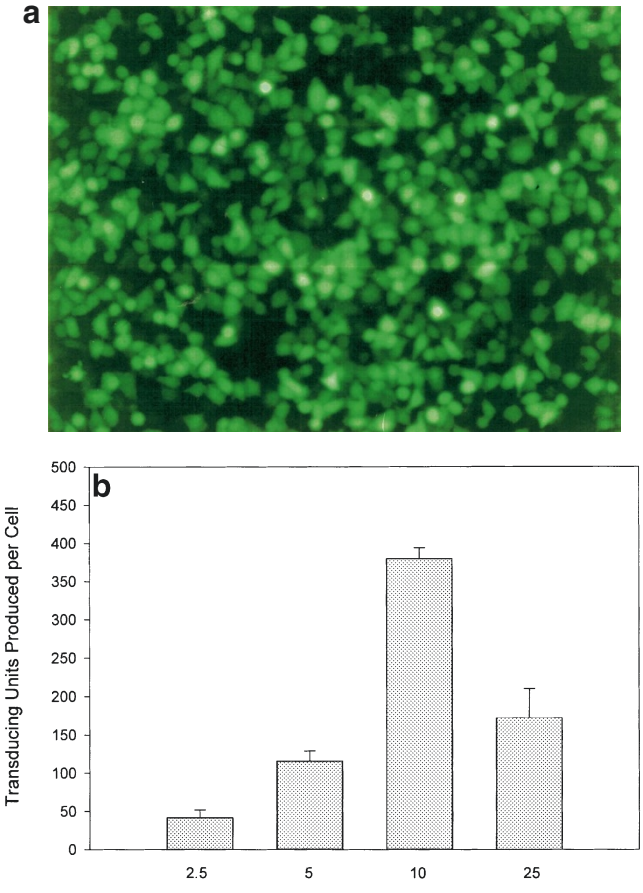

Figure 5 *(a) Purified AAV-GFP produced by d27.1-rc is infectious. C12 cells were infected with the AAV-GFP (MOI 5 EU) produced by d27.1-rc. The cells were then coinfected with Ad (MOI 20). Fluorescent microscopy was used to detect GFP expression 24 h after infection. Magnification ×100. (b) The vector d27.1-rc can efficiently produce rAAV from transfected 293 cells. 293 cells were transfected with AAV-GFP proviral plasmid. (Approximately 3×10^7 cells were present in each experimental group.) Twenty-four hours after transfection, the cells were superinfected with different MOIs of d27.1-rc. Thirty-six hours after infection, a cell lysate was made from the infected cells by three rounds of freeze–thaw. The cell lysate was heat inactivated at 55°C for 1 h and then titered in duplicate on C12 cells that were co-infected with Ad (MOI 20). Forty-eight hours after infection the C12 cells were analyzed for GFP expression using fluorescent microscopy and a titer was determined (in EU). The amount of AAV-GFP produced per transfected cell was then calculated. The data represent triplicate experiments.*

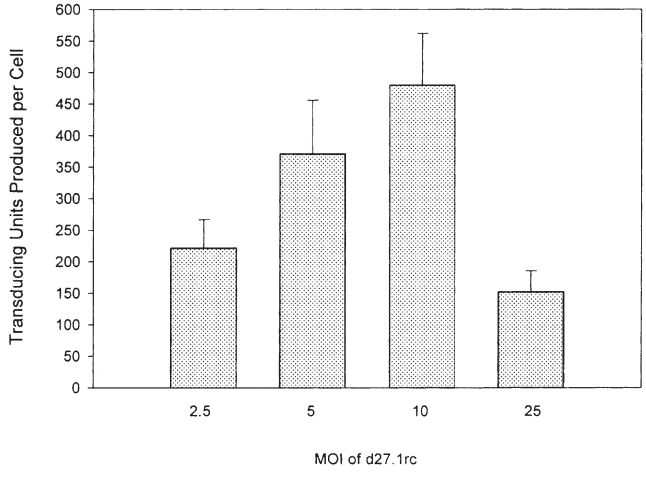

Figure 6 *The vector d27.1-rc can produce rAAV from an AAV-GFP proviral cell line. The cell line GFP-92 is a 293-derived cell line that has a single copy of AAV-GFP integrated into its genome. The vector d27.1-rc was used to produce AAV-GFP from this cell line. 1.5×10^7 GFP-92 cells were infected with d27.1-rc at different MOIs. Forty-eight hours after infection a cell lysate was made from the infected cells by three rounds of freeze–thaw. The cell lysate was heat inactivated at 55°C for 1 h and then titered in duplicate on C12 cells that were co-infected with Ad (MOI 20). Forty-eight hours after infection the C12 cells were analyzed for GFP expression using fluorescent microscopy and a titer was determined (EU). The amount of AAV-GFP produced per transfected cell was then calculated. The data represent triplicate experiments.*

(Table 1). When rAAV and rHSV are co-infected in 293 cells, amplification of rAAV genomes is observed. Infection with *d27.1-rc* (MOI of 10) along with rAAV (MOI = 0.1) leads to a 200-fold amplification of input AAV-GFP. The total amplification of rAAV was greater than 10^6-fold after three cycles of passage. While not as efficient as the production of AAV-GFP from transfected plasmid or a proviral cell line, co-infection of rAAV vectors with *d27.1-rc* will permit serial amplification of rAAV via scaleable infection.

The efficiency of rAAV production by d27.1-rc is maintained when the scale of production is increased

To determine if *d27.1-rc* can be utilized to produce rAAV on a larger scale, 10^9 GFP-92 cells were infected with *d27.1-rc* (Table 2). The yield of AAV-GFP, 380 EU / cell and 338 EU / cell in duplicate experiments, indicates that *d27.1-rc* is still able to efficiently produce rAAV after the

Table 1 Serial passage of rAAV with *d27.1-rc* results in vector amplification

Passage No.	*Input vector*	*Output vector*	*Fold amplification*	*Total amplification*
1	5.0×10^3	1.0×10^6	200	200
2	1.0×10^4	1.75×10^6	175	3.5×10^4
3	1.75×10^4	2.97×10^7	170	5.95×10^6

Recombinant adeno-associated virus can be amplified after co-infection with *d27.1-rc*. 293 Cells were infected with different MOIs of AAV-GFP as indicated. Twelve hours after infection, the cells were superinfected with *d27.1-rc* at a MOI of 10. Forty-eight hours after infection a cell lysate was made from the infected cells by three rounds of freeze–thaw. The cell lysate was heat inactivated at 55°C for 1 h and then titered in duplicate on C12 cells that were co-infected with adenovirus (MOI 20). Forty-eight hours after infection the C12 cells were analyzed for GFP expression using fluorescent microscopy and a titer was determined (EU). The data represent duplicate experiments.

Table 2 Efficient large-scale production of rAAV is observed using *d27.1-rc*

Experiment No.	*No. of GFP-92 cells*	*Amount of virus produced in cell lysate (EU)*	*EU produced per cell*
1	1.0×10^9	3.8×10^{11}	380
2	1.1×10^9	3.7×10^{11}	338

scale of infection is increased. Maintaining efficient rAAV production as the scale of *d27.1-rc* infection is increased is required if *d27.1-rc* is a viable helper for large-scale production of rAAV.

Discussion

Recombinant adeno-associated virus-mediated gene transfer has been uniquely successful in achieving long-term, high-level gene expression *in vivo*. Many potential applications for the use of rAAV in genetic disease will require a substantial vector dose to achieve a therapeutic effect. One significant problem associated with rAAV vectors, has been the difficulty in generating sufficient quantities of high-titer vector required for *in vivo* applications. This difficulty has led to improvements in numerous aspects of rAAV vector development in order to increase the efficiency of rAAV production. These strategies have all involved the use of adenovirus to provide the helper functions for rAAV production, however. Few studies have explored the possibility of using other helper viruses of AAV-2 replication and packaging for large-scale production.

In this paper, we report the development of an alternative system for production of rAAV. This system is based upon the HSV-1 helper functions of AAV-2 replication and packaging. By generating a recombinant HSV-1 encoding the AAV-2 *rep* and *cap* genes we have made a single infectious helper. The expression of Rep from this vector appears to be regulated and is appropriately distributed to the nucleus. The rHSV-1, *d27.1-rc*, propagates readily and its replication is not affected by the presence of *rep*.

Development of mature HSV-1 replication centers in the presence of *rep* appears to be unique to this vector. One possible explanation why the presence of the *rep* gene did not affect the kinetics of *d27.1-rc* replication or the formation of mature viral replication centers is that Rep proteins are not efficiently expressed in the V27 cells after *d27.1-rc* infection. Both Western analysis and an IFA were used to analyze Rep expression in 293, Vero and V27 cells after *d27.1-rc* infection. By Western analysis, high level Rep expression was observed in 293 cells and Vero cells but not in V27 cells after infection with *d27.1-rc* (Figure 2). By IFA, Rep expression was observed in the nucleus of infected 293 cells (Figure 3a) and Vero cells (data not shown) after infection with *d27.1-rc*, but not in V27 cells (Figure 4b). The minimal Rep expression after *d27.1-rc* infection of V27 cells may explain how generation of *d27.1-rc* was feasible and why similar efforts to construct recombinant Ad vectors with the *rep* gene have failed.

The *d27.1* vector was chosen as the mutant background to provide the viral helper functions for several reasons. The vector *d27.1* has a mutation in the immediate–early gene IE63 and does not produce ICP27.[31] The protein ICP27 has been implicated in the inhibition of host cell mRNA splicing.[47,48] The use of *d27.1* should minimize inhibition of splicing of the *rep* and *cap* messages compared with a vector which produces ICP27. In addition, *d27.1* overexpresses ICP8,[31] one of the HSV-1 genes essential for AAV-2 replication.[28] High-level expression of ICP8, the single-stranded DNA binding protein, should be beneficial for rAAV production.

The most efficient manner in which *d27.1-rc* can be used for large-scale rAAV production involves infection of a proviral cell line that provides the rAAV template to be packaged. In this two-component system, the proviral cell line could be grown at high densities in large quantities in spinner cultures or cartridge systems. The AAV-2 *rep* and *cap* genes and the helper functions required for rAAV production are then provided by *d27.1-rc* infection. Using *d27.1-rc* to infect the proviral cells would eliminate the need for transfection at any step in the production process. The choice of cell line used for this system is important, however. The results of Western analysis (Figure 2) indicate that *d27.1-rc* will efficiently express the AAV-2 Rep proteins only in certain cell lines.

The dose-response curve for the production of AAV-GFP by *d27.1-rc* demonstrates that increasing the MOI of *d27.1-rc* augments rAAV production to a point. The vector *d27.1-rc* still expresses the immediate–early genes that encode the viral proteins ICP0 and ICP4.[31] Expression of these immediate–early genes is detrimental to the cell and induces cell death.[49,50] At high MOIs, increased expression of these immediate–early genes probably leads to rapid cell death, limiting the production of rAAV. At a MOI of 25, while there is increased expression of the AAV-2 *rep* genes and the HSV-1 helper genes necessary for rAAV production, increased cytotoxicity due to additional gene expression from the vector also occurs. At a MOI of 10, the most effective balance exists between expression of the AAV-2 *rep* and *cap* genes and HSV-1 helper functions required for rAAV production and the cytotoxicity inherent to the vector.

Replication of HSV-1 is not required for efficient repli-

cation and packaging of AAV-2.[51] Cells lines such as 293 cells, which do not complement *d27.1-rc* replication, can therefore be used to produce rAAV. Using a non-complementing cell line to produce rAAV will permit the production of rAAV without generating additional *d27.1-rc*. The helper virus, *d27.1-rc*, will therefore be effectively eliminated from the rAAV produced.

The application of a recombinant virus to introduce the AAV-2 *rep* and *cap* and helper virus functions into cells to produce rAAV has many advantages over an amplicon system which we previously described.[32] Unlike a recombinant HSV-1 vector, an amplicon system has a variable helper virus to amplicon virus ratio from passage to passage. This variability makes optimization of an amplicon system for rAAV production difficult since the ratio of helper virus to amplicon virus will affect the amount of rAAV produced. In addition, there is no selective pressure to maintain the recombinant AAV-2 genome in the amplicon. With passage, deletion and recombination of the amplicon genome is likely to occur, resulting in decreased efficiency of rAAV production after serial passage of the amplicon. These problems are not encountered using the recombinant virus *d27.1-rc*.

Large-scale production of rAAV vectors will be required for *in vivo* preclinical and clinical trials of potentially therapeutic rAAV vectors. The vector *d27.1-rc* should facilitate the production of rAAV. The vector *d27.1-rc* is flexible and can be utilized to produce rAAV from transfected cells, cell lines or even infected rAAV. The rescue of rAAV from proviral cell lines at or above the efficiency of Ad-free methods will permit large-scale production of rAAV without requiring a transfection procedure. Combined with recently developed purification procedures (see accompanying article in this issue, pp 973–985), *d27.1-rc* will be an attractive way to produce the large quantity of rAAV that will be needed for clinical success of rAAV-based gene therapy.

Materials and methods

Plasmids

The plasmid pTR-UF5 is an AAV-GFP proviral construct with AAV-2 ITRs flanking both an eGFP and a neomycin resistance gene (*neo*) expression cassette. Expression of GFP is driven by the human CMV promoter. The *neo* gene is expressed from the HSV-1 *tk* promoter. The plasmid pSub201 contains the AAV-2 *rep* and *cap* genes and has previously been described.[16] The plasmid pHSV-106 is a pBR-derived plasmid into which the *Bam*HI fragment of HSV-1 (17+ stain) containing the thymidine kinase (*tk*) gene was cloned. The plasmid pHSV-106-*lacZ* was constructed by cloning a *lacZ* expression cassette into the *Kpn*I restriction site of pHSV-106 interrupting the *tk* gene. The plasmid pHSV-106-rc has the AAV-2 *rep* and *cap* genes from pSub201 cloned into the *Kpn*I site of pHSV-106.

Cell lines

The 293 and Vero cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). The V27 cell line is a Vero-derived cell line that expresses the HSV-1 ICP27 protein and has previously been described.[31] The C12 cell line is a Hela-derived cell line with inducible AAV-2 *rep* gene expression and has been previously described.[22] The GFP-92 cell line was created by infecting 293 cells with AAV-GFP and has been previously described.[32] In AAV-GFP, expression of GFP is driven by the human CMV promoter and the *neo* gene is expressed from the HSV-1 *tk* promoter. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS).

HSV-1 viruses

The virus *d27.1* is an ICP27 deletion mutant (Kos strain) which has been previously described and is propagated on the complementing cell line, V27.[31] The virus *d27.1-rc* was constructed by first creating the *lacZ* expressing virus *d27.1-lacZ*. The *lacZ* expressing vector was created by traditional techniques involving cotransfection of *d27.1* infected cell DNA and the integrating plasmid, pHSV-106-*lacZ* (linearized by *Bam*HI restriction digest) into V27 cells. Recombinant viruses were isolated by screening for blue plaques after agar overlay containing 400 μg/ml halogenated indolyl-β-d-galactoside (Bluogal; Gibco-BRL, Bethesda, MD, USA). Recombinant viruses were purified by three rounds of limiting dilution. Integration was confirmed by Southern analysis of restriction enzyme digested *d27.1-lacZ* infected cell DNA (data not shown). The virus *d27.1-rc* was created by cotransfection of *d27.1-lacZ* infected cell DNA and the *Sph*I linearized integration plasmid pHSV-106-rc into V27 cells. Recombinant viruses were isolated by screening for white plaques after agar overlay containing 400 μg/ml Bluo-gal. Recombinant viruses were purified by three rounds of limiting dilution. Integration was confirmed by Southern analysis of restriction enzyme digested *d27.1-rc* infected cell DNA (data not shown). The stability of integration with passage was assessed by isolating 10 clones of *d27.1-rc* after 10 serial passages of *d27.1-rc* at a MOI of 0.1. All clones were able to rescue rAAV (data not shown). Wild-type HSV-1 virus capable of replicating on Vero cells was not detected in any preparation (limit of detection is <20 plaque forming units (p.f.u.)/ml).

Recombinant AAV production methods

Production of AAV-GFP from pTR-UF5 transfected 293 cells: Tissue culture dishes (10 cm) plated with 2×10^6 293 cells were transfected with 5 μg pTR-UF5 and 25 μl Lipofectamine (Gibco-BRL) as per the manufacturer's instruction. Four hours after transfection, the cells were washed and DMEM (10% FBS) was added. Twenty hours later, the cells were superinfected with *d27.1-rc* at different MOIs or *d27.1-lacz* at a MOI of 10. (The cells on an extra transfected dish were trypsinized, resuspended and counted using a hemocytometer.) Approximately 3.5×10^7 cells were infected per MOI. Forty-eight hours later, the cells were harvested and pelleted by centrifugation (375 *g*, 5 min). The cells were then resuspended in 10 ml of DMEM and cell-associated rAAV was released by three rounds of freezing and thawing. Cell debris was pelleted by centrifugation (250 *g*, 5 min). The cell lysates were then titrated for EU of AAV-GFP as described below and purified by CsCl gradient as previously described.[1] This experiment was repeated in triplicate.

Production of AAV-GFP from the cell line GFP-92: The GFP-92 cells were plated in 75 cm^2 tissue culture flasks. Twelve hours later, the cells were infected with *d27.1-rc*

at different MOIs or *d27.1-lacz* at a MOI of 10. The number of cells in one extra flask was determined as described above. Approximately 1.5×10^7 GFP-92 cells were infected per MOI. Cells were harvested 48 h after infection and cell-associated AAV-GFP was processed and titered as described above. This experiment was repeated in triplicate.

Production of AAV-GFP by amplifying AAV-GFP via infection

293 Cells (1.5×10^6 cells) were plated in six-well tissue culture dishes. Twelve hours later, the cells were infected with AAV-GFP at different MOIs. Twelve hours later, the cells were infected with *d27.1-rc* at a MOI of 10. Cells were harvested 48 h after infection and cell-associated AAV-GFP was processed as described above. This experiment was repeated in triplicate. The amount of output rAAV was determined using the fluorescent cell assay described below.

Large-scale AAV-GFP production

GFP-92 cells were plated on 175 cm^2 tissue culture flasks 12 h before infection. 1×10^9 GFP-92 cells were infected 12 h later with *d27.1-rc* at a MOI of 10. Cells were harvested 48 h after infection and cell-associated AAV-GFP was processed as described above. This experiment was repeated in duplicate. Stocks were analyzed for replication-competent AAV (rcAAV) as previously described.[3] Replication-competent AAV was not detected (limit of detection was one replication unit per 10^7 GFP EU).

Titering of AAV-GFP in the cell lysates by the fluorescent cell assay

Cell lysates were heat inactivated (55°C, 1 h). Serial dilutions of AAV-GFP were then titered on C12 cells with Ad co-infection (MOI 20) as previously described.[52] The cells were then analyzed for GFP expression using fluorescence microscopy at 48 h after infection.

Western analysis of AAV-2 Rep proteins

The indicated cells (approximately 4×10^6 cells) were plated on to 6 cm tissue culture plates 12 h before infection with *d27.1-rc* (MOI as indicated). Control samples were not infected. Cells were harvested 48 h after infection and cell lysates were made and loaded on to a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel followed by immunoblotting using a monoclonal antibody (clone 1F11.8, 1:5000 dilution) that recognizes all four AAV-2 Rep proteins. The antibody was detected by chemiluminescence (Amersham, Arlington Heights, IL, USA).

Immunofluorescence assay

Cells (293, Vero or V27 cells) were plated on to two-well tissue culture slides at a density of 1.5×10^5 cells per well. For the anti-AAV Rep immunofluorescence assay, 293 cells were infected 12 h later with *d27.1-rc* at a MOI of 10. Cells were washed with DMEM after a 45 min adsorption period and DMEM with 10% FBS was then added. After 10 h, cells were washed twice with PBS and fixed for 10 min in 4% paraformaldehyde in PBS. Cells were washed twice with PBS and permeabilized with 0.2% Triton X-100 in PBS for 2 min. Cells were then washed twice with PBS and incubated for 1 h at 37°C in a humidified chamber with monoclonal anti-Rep antibody (American Research Products, Belmont, MA, USA, clone 226.7, 1:1 dilution). This antibody recognizes all four Rep proteins. The cells were then washed three times with PBS and incubated for 30 min at 37°C with FITC-conjugated, donkey anti-mouse secondary antibody (diluted 1:100 in 2% goat serum, 2% donkey serum in PBS). The slides were then washed three times, covered with a 4′, 6-diamidino-2- phenylindole (DAPI) containing mounting solution (Vector Laboratories, Burlingame, CA, USA), sealed and analyzed for immunofluorescence. Microscopy was performed on a Leitz microscope with Image Pro acquisition equipment and image analysis software.

To analyze the maturation of HSV-1 viral replication centers and Rep expression in V27 cells after *d27.1-rc* infection, a rabbit polyclonal anti-ICP8 (the HSV-1 single-standed DNA binding protein) antibody (PAb 3–83) and the monoclonal anti-Rep antibody (American Research Products, clone 226.7, 1:1 dilution) were utilized in a double label experiment. All procedures were as previously described except that V27 cells were infected at a MOI of 1. After fixing and permeablization, V27 cells were incubated as above with the anti-Rep monoclonal antibody. The cells were then washed twice with PBS and incubated with the anti-ICP8 antibody (diluted 1:50 in 2% goat serum, 2% donkey serum in PBS) for 1 h in a humidified chamber at 37°C. The cells were then washed three times with PBS and incubated with a rhodamine-conjugated, donkey anti-rabbit secondary antibody and FITC-conjugated, donkey anti-mouse secondary antibody (both diluted 1:100 in 2% goat serum, 2% donkey serum in PBS) for 30 min at 37°C. The slides were then washed three times, covered with DAPI containing mounting solution, sealed and analyzed for immunofluorescence. (Vero cells were infected and processed alongside V27 cells to serve as positive controls for Rep staining.)

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